THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

# RON'S INVOLVEMENT IN THE INFLAMMASOME PATHWAY ATTENUATES ALZHEIMER'S-ASSOCIATED NEUROINFLAMMATION

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# ABSTRACT

Out of the top 10 causes of death in the United States, Alzheimer's Disease (AD) is the only one that currently cannot be prevented, slowed, or cured. Ongoing research to unravel this complex and devastating illness has shown that a chronic state of inflammation contributes to worsening pathological conditions of AD and may even provoke an earlier onset of the disease. Therefore, understanding the cellular signaling pathways involved in mitigating inflammation in the brain could elucidate new potentials for preventative therapies or novel drug targets in AD. One pathway of interest involves the RON receptor tyrosine kinase (RON) expressed on macrophages. Previous studies have demonstrated that RON knockout mice exhibit more inflammation than their wild type counterparts, suggesting that RON plays a protective role in attenuating the inflammatory state. Although we know that RON is at the beginning of a pathway that quells inflammation, the downstream events of this pathway are largely unknown. Therefore, this study aimed to elucidate the downstream regulatory effects of RON by establishing a connection between RON and another hallmark inflammatory pathway known to occur in macrophages. We hypothesized that RON was involved in suppressing signal 2 of the inflammasome pathway, thus limiting inflammasome activation. Using an atherosclerosis model to simulate a chronic inflammatory state, whole brains and brain regions were harvested from control and RON-KO mice and analyzed for expression levels of key inflammasome-pathway proteins (including NLRP3, IL-1β, and caspase 1) using western blot analysis. The results show that, in the whole brain, hippocampus, and hypothalamus, RON-KO mice express higher levels of NLRP3, cleaved caspase 1 and cleaved IL-1β, indicating that RON plays a role in suppressing not only the expression of these inflammasome proteins, but also their activation. These novel results are the first to demonstrate RON's involvement in signal

2 of the inflammasome pathway in the brain, and shed new light on the function of the RON receptor and its potential to alleviate the inflammatory state implicated in the early stages of AD.

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## ACKNOWLEDGEMENTS

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# Chapter 1

## Introduction

Out of the top 10 causes of death in the United States, Alzheimer's Disease (AD) is the only one that currently cannot be prevented, slowed, or cured.[3] This devastating neurodegenerative disease is characterized by the destruction of neurons in the brain, leading to severe atrophy in regions such as the hippocampus, resulting in memory impairment.[5] This neurodegeneration and subsequent brain atrophy is the consequence of cerebral inflammation, which is exacerbated by the presence of abnormal protein aggregates which promote an inflammatory response. However, ongoing research has demonstrated that chronic inflammation in the brain earlier in life, brought on by environmental and lifestyle factors such as obesity, contributes to worsening pathological conditions of AD and may even provoke an earlier onset of the disease.[3] Because inflammation underlies the pathology of AD, it is important to not only understand which cells in the brain contribute to this inflammatory state, but also to consider mechanisms by which this inflammation is attenuated in an effort to slow or perhaps even prevent the progression of such a fatal and prevalent disease.

## Macrophages and Inflammation

Macrophages, a subpopulation of innate immune cells, are known to be key players in inflammatory processes throughout the body. These cells exist in a balance between two opposing polarization states: M1 and M2. Normally, in healthy tissue free from injury or disease, macrophages express the M2 phenotype, which tends to be more reparative in nature and

contributes to day-to-day tissue homeostasis and resolution of inflammation.[1] However, upon encountering tissue damage or disease, resident macrophages become activated and shift to the M1 phenotype, which is regarded as proinflammatory and produces mediators that lead to inflammation.[4] Thus, by interconverting between these opposing states, macrophages are capable of both creating and resolving inflammation.[2] In the central nervous system (CNS), resident macrophages are known as microglia. This population of yolk-sac-derived macrophages are also capable of existing and interconverting between M1 and M2 phenotypes.[7] In the brain, microglia are highly sensitive to signs of disease and disruptions in homeostasis, and will respond to a disturbance by adopting an M1, classically-activated phenotype.[7] This M1 state promotes an inflammatory state within the CNS, which can aid in the resolution of disease states such as infection. However, if this neuroinflammatory state persists, it can actually do more harm than good, eventually resulting in neurodegeneration and tissue atrophy, which are observed in AD.[8] It has been demonstrated that a reduction in the M2 phenotype and an expansion of the M1 phenotype in the brain is associated with increased neuroinflammation, indicating that shifting the balance away from the M1 state and towards the M2 state could serve as a therapeutic strategy to reduce neuroinflammation and consequently, neurodegeneration observed in AD.[2]

## The Ron Receptor Tyrosine Kinase

Excitingly, one key receptor found on macrophages, including microglia, has been shown to do just that.[2] This receptor, known as the Ron receptor tyrosine kinase (RON), is one of the first proteins in a signaling cascade that functions to quell inflammation by inhibiting the expression of pro-inflammatory markers, such as IL-1β and IL-18 and promoting the expression of anti-

inflammatory molecules, such as Arginase-1 (Arg-1). When bound by its ligand, macrophage stimulating protein (MSP), RON has been shown to both promote an anti-inflammatory M2 phenotype and limit the M1 pro-inflammatory phenotype.[1] Knockout studies, whereby the ligand binding domain of RON was deleted to produce a non-functional receptor (RON KO), have also demonstrated that the loss of a functional RON receptor exacerbates inflammation by promoting M1 macrophage activation and hindering M2 macrophage activation, suggesting that RON plays a protective role in attenuating a neuroinflammatory state.[2] However, the complete downstream mechanism by which RON quells inflammation is yet to be characterized.

## RON and the NLRP3 Inflammasome

The inflammasome is a signaling complex composed of multiple proteins, including ASC, NLRP3, and pro-caspase 1, found in myeloid cells, including macrophages, where it functions as a component of the innate immune response.[9] The end goal of inflammasome activation is to promote the cleavage and activation of pro-caspase 1, thus promoting caspase-1-mediated cleavage of the proinflammatory cytokines, IL-18 and IL-1β.[10] Activation of the NLRP3 inflammasome is regulated in two distinct stages which occur in response to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs):

1. Signal 1: Transcriptional Regulation - In this first stage, a PAMP or DAMP is recognized by a cellular receptor, leading to the activation of NFkB, a transcription factor. Once translocated into the nucleus, NFkB serves to upregulate the transcription of key inflammasome components including NLRP3, pro-caspase 1, pro-IL-1β and pro-IL-18.[10]

2. Signal 2: Post-Translational Regulation - In this stage, a second signal is required to promote assembly of these proteins and to activate the inflammasome. This signal again arises from receptor recognition of a PAMP or DAMP, including ATP, reactive oxygen species (ROS), or β-amyloid. Once activated, the inflammasome promotes cleavage of pro-caspase 1, which then cleaves pro-IL-1β and pro-IL-18 into their active forms, which are then secreted.[10]

Previously, it has been demonstrated that RON plays an inhibitory role in the inflammasome pathway. Past studies in periphery macrophages have shown that RON interferes with signal 1 of inflammasome activation by inhibiting the activation of NFkB, thus preventing it from translocating into the nucleus and thereby limiting the expression of key inflammasome proteins including NLRP3, pro-caspase 1, pro-IL-1β and pro-IL-18.[1]

Up until this point, there has been no evidence implicating RON in the inhibition of signal 2. However, preliminary data from our lab, in combination with previous studies, indicate that RON plays a role in downregulating the production of ROS, a key DAMP involved in signal 2 activation.[11] Thus, we hypothesized that, in addition to suppressing transcription of inflammasome genes in signal 1, RON also plays a role in suppressing the post-translational activation and cleavage of inflammasome proteins in signal 2, thereby limiting the M1, pro-inflammatory microglial phenotype.

# Chapter 2

# Materials and Methods

## Mice

Male apolipoprotein E knockout (ApoE KO - henceforth known as "control") mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME, USA). These male apolipoprotein E knockout mice were crossed with existing RON KO mice from our lab to create male apolipoprotein E knockout + RON knockout mice on a C57BL/6 background (DKO - henceforth known as "RON KO"). The RON KO was formed by a deletion of the receptor's ligand binding domain. The following experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at The Pennsylvania State University (#46345).

## Diets

Previously, our lab has shown that a high-fat diet can model a state of chronic neuroinflammation similar to that observed in chronic neurodegenerative diseases.[2] Thus, to model a state of chronic CNS inflammation, both 6-week old control and RON KO mice were placed on a high-cholesterol + high-fat diet (HFHCD) for 18 weeks (F6334-Bioserve 60% high-fat and 1.25% high-cholesterol chow) to stimulate vascular and severe tissue inflammation in this murine atherosclerosis model.

## Dissection

Mice were euthanized by $CO_2$ narcosis. Following euthanasia, whole brains and brain sections (including hypothalamus, hippocampus, frontal cortex, and hindbrain) were harvested from control and RON KO mice following 18 weeks of diet-induced disease.

## Protein Extraction

Brain tissues were homogenized in an MPER solution (Thermo-Fisher) containing protease inhibitors as per product instructions.

## Western Blots

Lysates were fractionated on 12% (Caspase 1, IL-1β) or 10% (NLRP3) SDS-polyacrylamide gels followed by electroblotting onto PVDF membranes (Bio-Rad). Blots were probed with appropriate antibodies: IL-1β Polyclonal Antibody (Bioss), NLRP3 Polyclonal Antibody (gifted from Alnemri Lab - Sidney Kimmel Medical College, Philadelphia PA), and Caspase 1 Polyclonal Antibody (gifted from Alnemri Lab - Sidney Kimmel Medical College, Philadelphia PA). Relative density of blots was quantified using ImageJ.

## Statistical Analysis

Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired Student's t-test, paired Student's t-test, and one-way or two-way ANOVA. Differences were considered

significant at $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). All analysis was performed using GraphPad Prism 5.0 (San Diego, CA, USA).

# Chapter 3

## Results

### Experiment 1 – Whole Brains

In order to determine if RON does in fact play a role in suppressing the activation of inflammasome proteins via signal 2, whole brains were extracted from control and RON KO mice and analyzed for the expression of key inflammasome components, including NLRP3, cleaved caspase 1, and cleaved IL-1β, following 18 weeks of a high-fat, high-cholesterol diet to stimulate severe vascular and tissue inflammation in this murine atherosclerosis model.

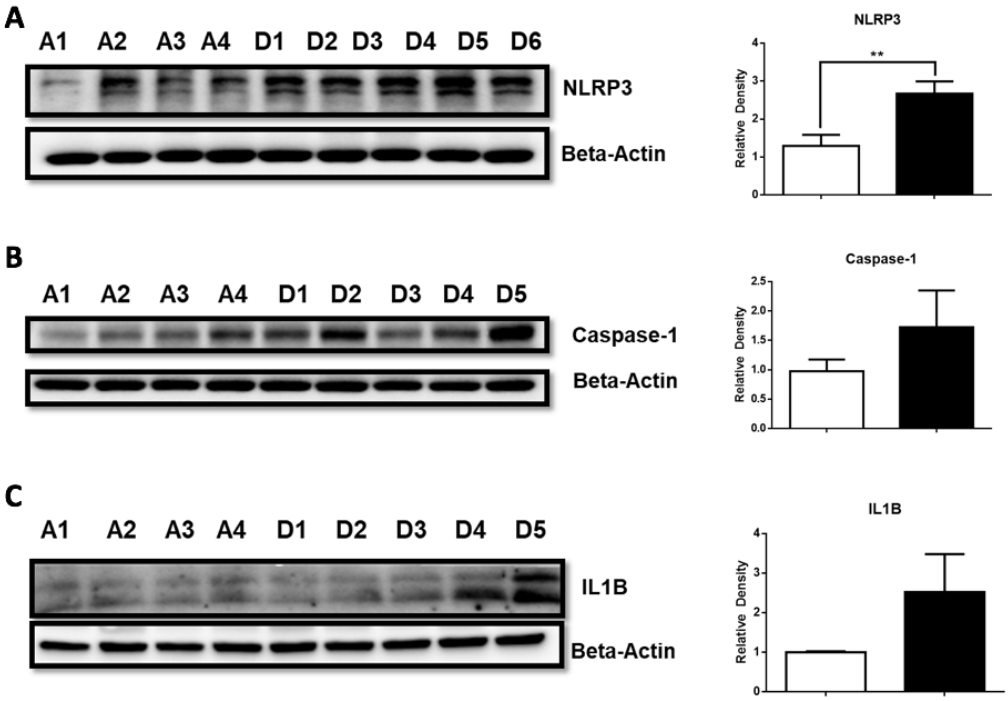



**Figure 1 Loss of RON function results in upregulation of activated inflammasome proteins in whole brain**

*Whole brains from RON KO mice show (A) a significant increase NLRP3 (p=0.01), (B) a trend increase in activated caspase-1, and (C) a trend increase in IL-1β, compared to control group. Left panel shows blots with β-actin as a control, right panel shows quantification of relative density to control β-actin in both RON KO and control groups. A1-4 represent samples from control group, D1-6 represent samples from RON KO group.*

As predicted, RON KO brains showed increased levels of all 3 inflammasome components: a significant increase in NLRP3, and trend increases in cleaved caspase 1 and cleaved IL-1β compared to brains from the control group.

## Experiment 2 – Brain Regions

In experiment 1, an increase in the expression and activation of inflammasome proteins was observed in the brain as a whole. However, microglia are known to show variations in gene expression based on the region of the brain in which they reside.[12] Therefore, in order to analyze the distribution of inflammasome activation throughout the brain, regional analysis of inflammasome protein expression was performed via western blot on areas including the hippocampus (figure 2), the hypothalamus (figure 3), the frontal cortex (sup. figure 1), and the hindbrain (sup. figure 2).

### *Hippocampus mirrors whole brain in expression and activation of inflammasome proteins*

The hippocampus is the region of the brain where neurodegeneration is classically studied. Inflammation-induced atrophy of this region is also regularly observed in AD, and accounts for the memory impairment that is an integral symptom of this disease.[5] Interestingly, as seen in figure 2, the expression and activation of inflammasome proteins in the hippocampus closely mirror that of the whole brain.

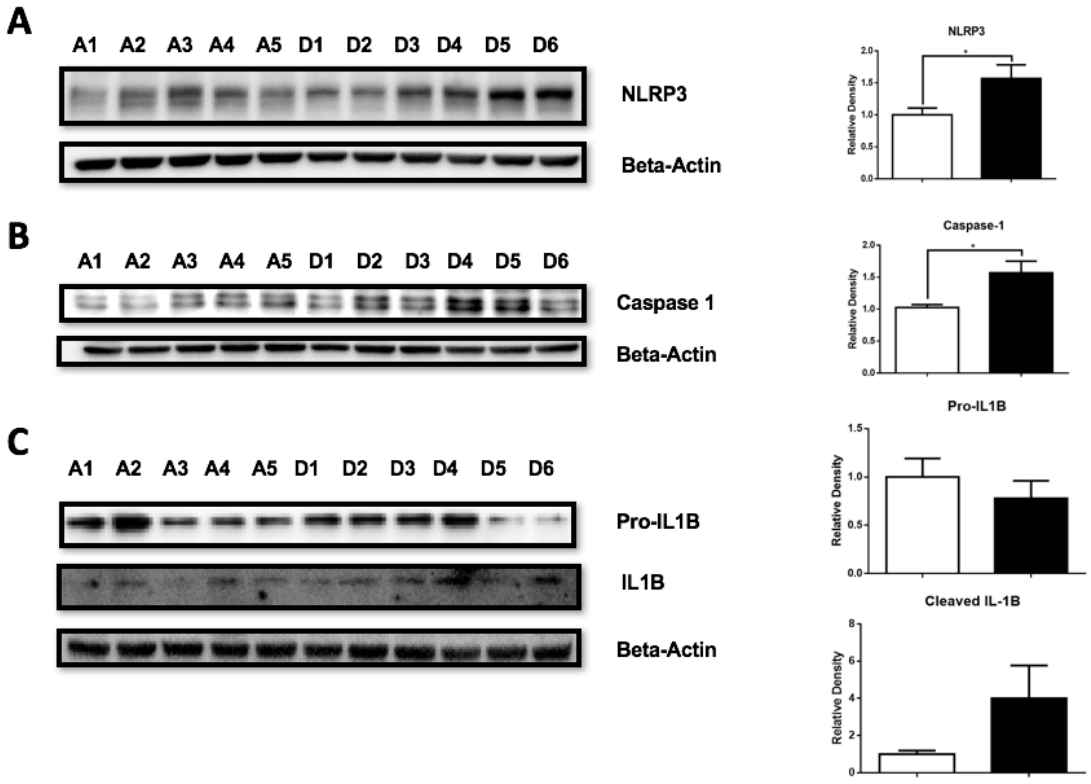



**Figure 2 Loss of RON function results in upregulation of activated inflammasome proteins in Hippocampus.**

*Hippocampi from RON KO mice show (A) significant increases in NLRP3 and (B) caspase 1 (p=0.05), (C) a trend decrease in pro-IL-1β, and a trend increase in cleaved IL-1β compared to control hippocampi. Left panel shows blots with β-actin as a control, right panel shows quantification of relative density to control β-actin in both RON KO and control groups. A1-5 represent samples from control group, D1-6 represent samples from RON KO group.*

In the hippocampus, we again observe a significant increase in NLRP3 expression and a significant increase in cleaved caspase 1 in RON KO compared to control. In contrast to the apparent decrease in pro-IL-1β in RON KO relative to control, we see an increase in cleaved IL-1β in RON KO.

### *Hypothalamus shows inflammasome activation, but to a lesser extent*

In contrast to the hippocampus, the hypothalamus is the region of the brain where inflammation is classically studied in diet-induced inflammation models based on its relation to satiety and leptin ghrelin signal management.[14] Although this region does not reflect the expression patterns of the whole brain as directly as the hippocampus, there is still evidence of increased inflammasome activation in RON KO.

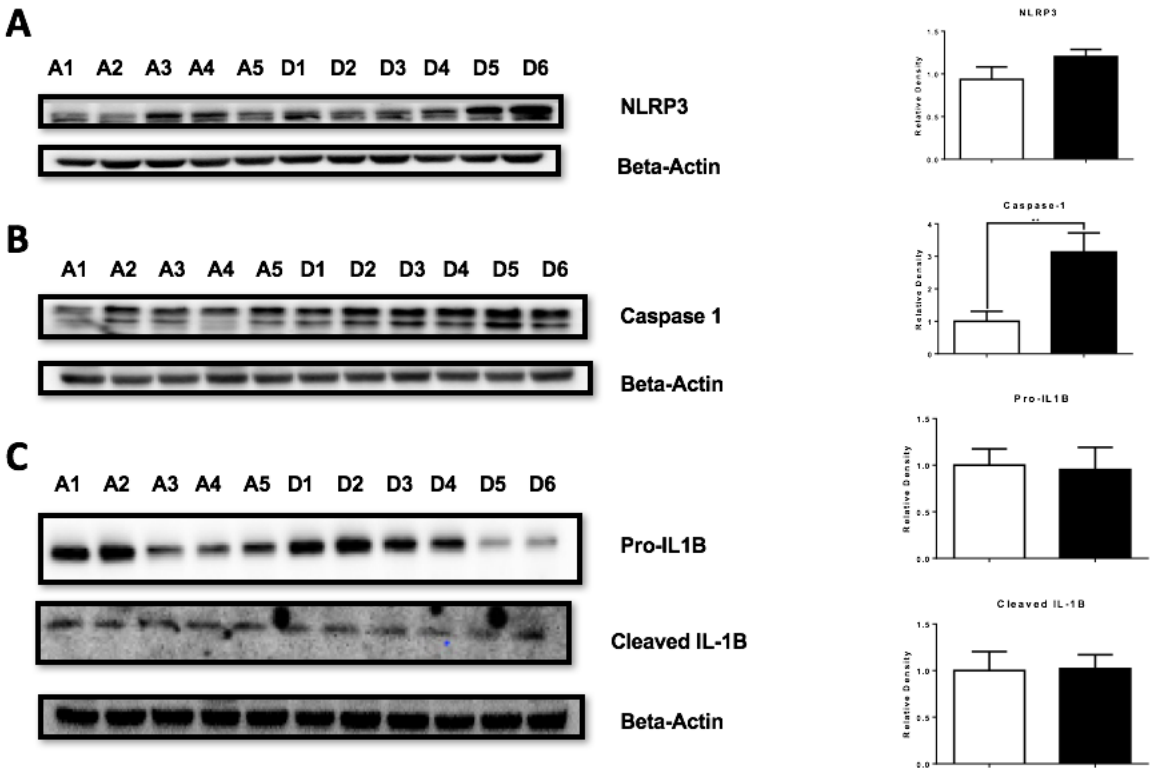



**Figure 3 Loss of RON function results in upregulation of activated inflammasome proteins in Hypothalamus.**

*Hypothalami from RON KO mice show (A) a trend increase in NLRP3, (B) a significant increase caspase 1 (p=0.01), (C) a trend decrease in pro-IL-1β, and a trend increase in cleaved IL-1β compared to control hypothalami.. Left panel shows blots with β-actin as a control, right panel shows quantification of relative density to control β-actin in both RON KO and control groups. A1-5 represent samples from control group, D1-6 represent samples from RON KO group.*

Compared to control, we see a trend increase in NLRP3, a significant increase in cleaved caspase 1, a slight trend decrease in pro-IL-1β, matched by a slight trend increase in cleaved IL-1β. These trends also reflect the expression and activation patterns observed in the hippocampus, however, in the hypothalamus, the differences between the control and RON KO groups is much less pronounced, with the exception of cleaved caspase 1.

# Chapter 4

## Discussion and Concluding Remarks

In accordance with the hypothesis, these results suggest that, in addition to suppressing transcription of inflammasome genes in signal 1, RON also plays a role in suppressing the post-translational activation and cleavage of inflammasome proteins in signal 2, thereby limiting the M1, pro-inflammatory microglial phenotype.

Firstly, this study confirmed the findings of previous studies which show that RON limits the transcription of inflammasome-associated genes like NLRP3 in step 1.[1] As seen in the whole brain, hippocampus, and hypothalamus, RON KO mice consistently express more NLRP3 compared to control mice. This trend can be explained by the fact that NLRP3 expression is under the transcriptional control of NFkB, and RON has been shown to restrict the nuclear localization of NFkB, thus limiting its ability to initiate transcription of inflammasome-associated genes and accounting for the increase in NLRP3 observed in mice without a functional RON receptor.[1]

Secondly, and perhaps most importantly, this study novely demonstrates that, in addition to its role in signal 1, RON also plays a role in signal 2 of the inflammasome pathway by limiting the activation of proteins involved in the inflammasome complex. In the whole brain, hippocampus, and hypothalamus, RON KO mice show higher levels of cleaved caspase 1 and cleaved IL-1β compared to control mice. Because the cleavage of these proteins is synonymous with their activation, higher levels of cleaved caspase 1 and cleaved IL-1β in RON KO brains demonstrate that increased activation of the inflammasome is occurring due to lack of a functional RON receptor. An additional trend that supports this conclusion is found in the discrepancy between

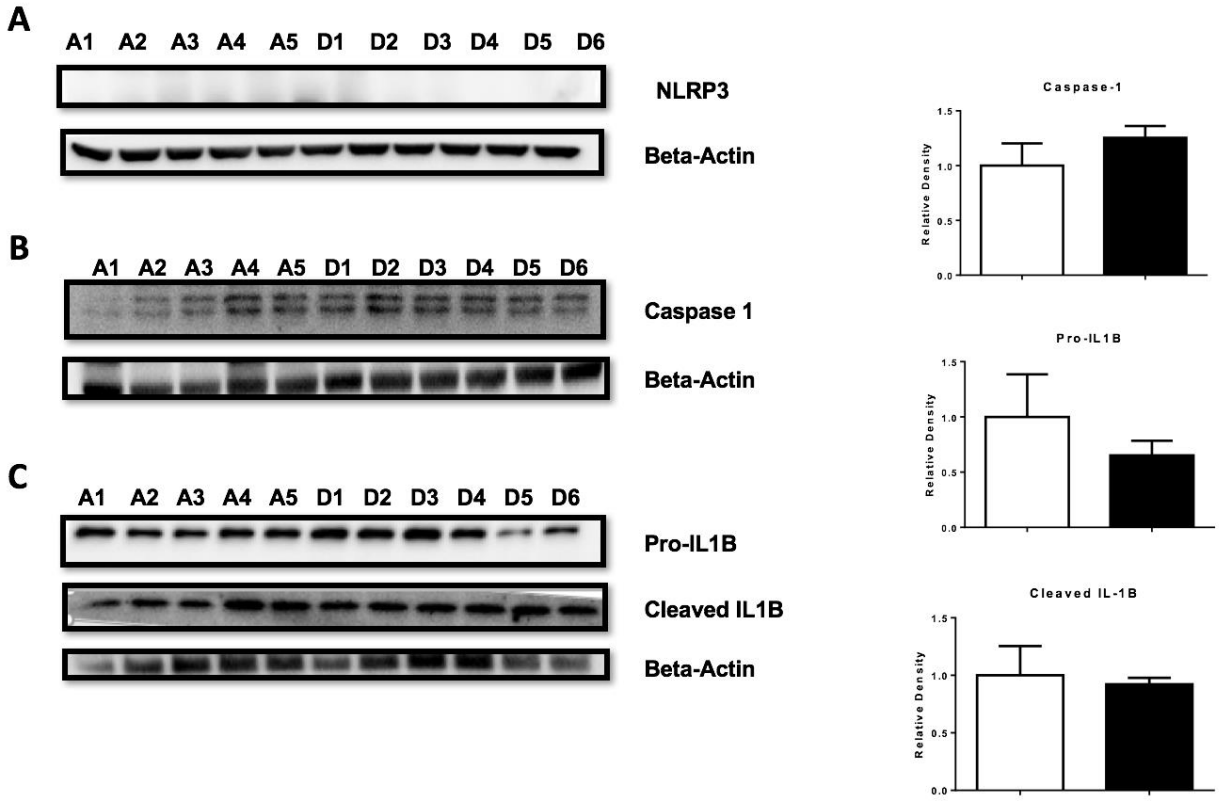
pro-IL-1β and cleaved IL-1β in control vs RON KO mice observed in the hippocampus and hypothalamus. In both of these regions, control mice express higher levels of pro-IL-1β than the RON KO group. At first, this seems contradictory because we would expect to see the opposite trend: in theory, the non-functional RON receptor found in the RON KO group should spur the upregulation of pro-IL-1β expression compared to control because RON is not there to inhibit the nuclear translocation of NFkB, the transcription factor that controls pro-IL-1β expression.[10] However, when pro-IL-1β expression in both groups is compared to cleaved IL-1β expression, it is apparent that this discrepancy may be explained by an increased conversion of pro-IL-1β to cleaved IL-1β in RON KO mice due to increased inflammasome activation. Thus, RON KO mice express less pro-IL-1β not because of a discrepancy in transcription, but rather due to increased cleavage of pro-IL-1β to cleaved IL-1β via activated caspase 1, which is also elevated in RON KO mice. Overall, these results highlight that increased activation of inflammasome proteins is occurring in RON KO brains compared to control brains, implicating RON as a receptor which normally functions to quell this activation by somehow suppressing signal 2.

In terms of how this suppression occurs, preliminary data from our lab, in combination with previous studies, indicate that RON plays a role in downregulating the production of reactive oxygen species.[15] Because reactive oxygen species are DAMPs known to trigger signal 2 activation,[13] we suspect that, mechanistically, RON suppresses signal 2 of inflammasome activation by downregulating the production of reactive oxygen species which would otherwise activate signal 2. Thus, by limiting the production of a signal 2 stimulus, RON indirectly limits the assembly and activation of the inflammasome, thereby limiting the M1, pro-inflammatory microglial phenotype.

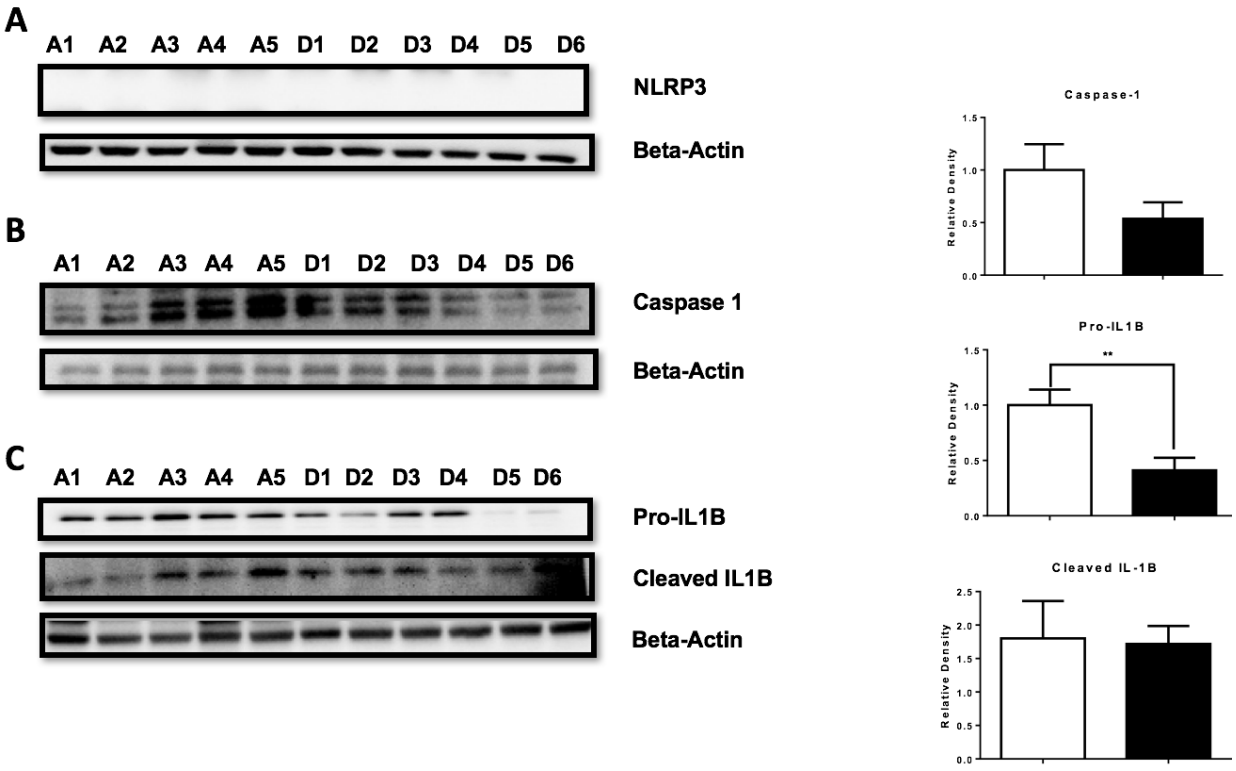
Overall, these novel results are the first to demonstrate RON's role in suppressing signal 2 in the activation of the inflammasome pathway in the brain, shedding new light on the function of the RON receptor and its potential to alleviate the inflammatory state implicated in the early stages of AD. We now know that a functional RON receptor limits the expression of the pro-inflammatory, M1 phenotype of microglia via the suppression of inflammasome activation in mice, and our next step is to see if RON serves this same role in human microglia. Currently, we are in the process of studying this pathway *in vitro* using a human CHME-3 microglial cell line. In order to better simulate the conditions observed in AD, we will be incubating cultures with amyloid β protein aggregates, which are a known DAMP stimulus for inflammasome activation in the brain.[13] These cells will also be treated with MSP, RON's agonist, in an attempt to show that activation of RON can downregulate inflammasome activation *in vitro*. If this pathway holds true in human microglia, it will further support the validity of RON as therapeutic target, with the hope that stimulation or upregulation of this receptor could serve not only as a potential therapeutic strategy to restore a healthy balance of M1:M2 microglia in the brain in current AD patients, but also as a potential preventative strategy to inhibit or at least slow the onset of this devastating disease before it starts, by attenuating a state of neuroinflammation earlier in life.

# Appendix A

## Supplemental Figures




**Supplemental figure 1: Frontal cortex.** Frontal cortices from RON KO mice show (A) no NLRP3 expression (B) a trend increase in caspase 1, (C) a trend decrease in pro-IL-1β and a trend decrease in cleaved IL-1β compared to control frontal cortices. Left panel shows blots with β-actin as a control, right panel shows quantification of relative density to control β-actin in both RON KO and control groups. A1-5 represent samples from control group, D1-6 represent samples from RON KO group.




**Supplemental figure 2: Hindbrain.** Hindbrains from RON KO mice show (A) no NLRP3 expression (B) a trend decrease in caspase 1, (C) a significant decrease in pro-IL-1β ($p=0.01$) and a trend decrease in cleaved IL-1β compared to control hindbrains. Left panel shows blots with β-actin as a control, right panel shows quantification of relative density to control β-actin in both RON KO and control groups. A1-5 represent samples from control group, D1-6 represent samples from RON KO group.

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## EDUCATION

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Bachelor of Science in Immunology and Infectious Disease ***Aug. 2014 – present***
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- Analyzed the inflammatory response and inflammasome pathway in murine cells
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**Remote Area Medical, Charleston, WV** ***Oct. 2017***

*Interpreter/General Volunteer*

- Communicated between medical volunteers and Spanish-speaking patients seeking free dental, vision, and general medical care at a weekend clinic
- Assisted with clinic logistics including patient intake and breakdown of medical supplies

## ACTIVITIES

**Schreyer Honors College Career Development Team** ***April 2015 – present***

*Lead Medical Event Coordinator*

- Organize 10-15 annual events involving medical professionals and alumni for 300+ students
- Lead a 5 member team to coordinate room reservations, food delivery, and speaker accommodations for each event
- Chaperone students during off-campus events and medical school treks, managing travel logistics and serving as a university representative

**Spanish Immersion Club** ***Aug. 2015 – May 2017***

*Founding Member and Cultural Outreach Director*

- Lead biweekly conversation nights by supplying topics of conversation and encouraging students to speak in Spanish
- Contact native speakers to attend conversation events and to teach students about their native cultures
- Prepare workshops on Spanish grammar, linguistics, and phonetics to help students improve their fluency, understanding, and appreciation of the Spanish language

**Penn State Anthropology Club** ***Aug. 2015 - present***

*Treasurer*

- Prepare annual club budgets, manage club finances, and participate in trainings with the Penn State Student Activity Office
- Co-direct meetings and plan events with other officers, exploring topics such as primitive pottery making and ethnomusicology

## PUBLICATIONS, POSTERS, & ACKNOWLEDGEMENTS

**Publications**

- Dey, A., **Hare, A.J.**, Fraser, J.W, Nettleford,S., Alnemri, D.M., Prabhu, K.S., Paulson, R.F., Hankey-Giblin, P.A. *MSP-dependent activation of CNS Ron receptor tyrosine kinase signaling attenuates neuroinflammation through the physiological regulation of IL1B and NLRP3 inflammasome pathway* (In prep-Journal of Cell Physiology/04-2018)
- Dey, A.. **Hare, A.J**, Hankey-Giblin,P.A. *Role of Macrophage Heterogeneity and Gender in obesity associated CNS inflammation*. (In Prep-AJP Integrated and Comparative Physiol)

**Posters**

- **Hare, A.J**., Hughes, D. *Colony Response to the Removal of Cadaver-Carrying Ants.* Gamma Sigma Delta Research Expo, 2017.
- **Hare, A.J**., Dey, A, Nettleford,S., Prabhu, K.S., Hankey-Giblin, P. A. RON's Involvement in the Inflammasome Pathway Attenuates Alzheimer's-Associated Neuroinflammation. Gamma Sigma Delta Research Expo, 2018.
- Adwitia Dey, Shaneice K. Nettleford, James W. Fraser, **Amelia J.Hare**, Diana M. Alnemri, Robert F.Paulson, K.Sandeep Prabhu and Pamela Hankey-Giblin. *MSP-dependent activation of CNS Ron receptor*

*tyrosine kinase signaling attenuates neuroinflammation through the physiological regulation of IL1B and NLRP3 inflammasome pathway. (*scheduled for 04/23/2018-Experimental Biology-San Diego)

**Acknowledgements**

- Dey, A.,Allen J.N., Fraser,J.W., Snyder,L.,Tian,Y., Zhang,L., Patterson,A., Cantorna, M.T., Paulson,R.F., Hankey-Giblin,P.A. *Neuroprotective Role of the Ron Receptor Tyrosine Kinase underlying CNS Inflammation in Health and Disease.* Accepted-02/2018-In Press-Frontiers in Immunol- 326540

## HONORS AND AWARDS

**University Honors Program**: Schreyer Honors College ***Aug. 2014 – present***

**Academic Excellence Scholarship**: $4,000 per year

**Gamma Sigma Delta Research Expo**: First prize recipient in Animal Sciences category 2017

## SKILLS

**Languages**

Spanish

- Fluent (Speaking, Reading, Writing)

Portuguese

- Proficient (Speaking, Reading, Writing)